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<b>(21) International Application Number:</b> PCT/GB94/00416 <b>(22) International Filing Date:</b> 4 March 1994 (04.03.94) <b>(30) Priority Data:</b> 104954                      4 March 1993 (04.03.93)                      IL <b>(71) Applicants (for all designated States except US):</b> YISSUM RESEARCH DEVELOPMENT COMPANY [IL/IL]; The Hebrew University of Jerusalem, 46 Jabotinsky Street, Jerusalem (IL). GOLDIN, Douglas, Michael [GB/GB]; 14 South Square, Gray's Inn, London WC1R 5LX (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BAB, Itai [IL/IL]; 26 Ha-teena Street, Carmei Joseph, M.P. Ayalon (IL). MUHLRAD, Andras [IL/IL]; 29/A Neve Shaanan Street, Jerusalem (IL). CHOREV, Michael [IL/IL]; 135/4 Feinstein Street, Jerusalem (IL). SHTEYER, Arie [IL/IL]; 37 Ha'arazim Street, Mevasseret Zion (IL). GREENBERG, Zvi [IL/IL]; 19 Joshua Ben-Nun Street, Givat Zeev (IL). MANSUR, Nura [IL/IL]; 3/24 Giborei Israel Street, 72462 Ramla (IL). <b>(74) Agents:</b> GOLDIN, Douglas, Michael et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, TJ, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> OSTEOGENIC GROWTH OLIGOPEPTIDES AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM		
<b>(57) Abstract</b> <p>The invention relates to biochemically pure oligopeptides having stimulatory activity on osteoblastic and/or fibroblastic cells having a molecular weight between 200 and 2,000. Preferred oligopeptides according to the invention comprise the amino acid sequences Tyr-Gly-Phe-His-Gly and Gly-Phe-Gly-Gly. The invention further relates to pharmaceutical compositions for the stimulation of formation of osteoblastic of fibroblastic cells, enhanced bone formation in osteogenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, or other conditions requiring enhanced bone formation cells comprising a therapeutically effective amount of an oligopeptide according to the invention.</p>		

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**OSTEOGENIC GROWTH OLIGOPEPTIDES AND PHARMACEUTICAL  
COMPOSITIONS CONTAINING THEM**

**Field of the Invention**

The invention relates to osteogenic growth oligopeptides which possess stimulatory activity on osteoblastic and fibroblastic cells.

**Background of the Invention**

- 5 It has been established that regenerating marrow induces an osteogenic response in distant skeletal sites and that this activity is mediated by factors released into the circulation by the healing tissue [Bab I. *et al* (1985) *Calcif. Tissue Int.* 37:551; Foldes, J. *et al* (1989) *J. Bone Min. Res.* 4:643; Einhorn, T.A. *et al* (1990) *J. Bone Joint Surg. Am.* 72:1374; Gazit D., *et al* (1990) *Endocrinology* 126:2607; Mueller, M. *et al* (1991)
- 10 *J. Bone Min. Res.* 6:401]. One of these factors, a 14-amino acid osteogenic growth polypeptide (OGP), identical with the C-terminus of histone H4, has been recently identified [Bab, I. *et al* (1992) *EMBO J.* 11:1867; European Patent Applications Nos. 89201608.0 and 90301862.0]. A histone H4 fragment of the formula Tyr-Gly-Phe-Gly-Gly is disclosed in Kharchenko, E.P. *et al* (1989) *Vopr. Med. Khim.* 35(2):106-9 and in
- 15 Kharchenko, E.P. *et al* (1987) *Biull. Eksp. Biol. Med.* 103(4):418-20. This peptide demonstrated analgesic and opiod activity.

- Synthetic 14-mer osteogenic growth polypeptide (sOGP), identical in structure with the native molecule, has been shown to be a potent stimulator of proliferation of osteoblastic and fibroblastic cells *in vitro*. This synthetic polypeptide also stimulates
- 20 osteoblastic cell alkaline phosphatase activity. When injected *in vivo* to rats, at very small doses, the synthetic osteogenic growth polypeptide increases bone formation and trabecular bone mass.

- As in the case of other polypeptide growth regulators, such as growth hormone and insulin-like growth factor [Hintz, R.L. (1990) *Horm. Res.* 33:105], osteogenic growth
- 25 polypeptide-binding protein/s (OGPBP) may protect the osteogenic growth polypeptide against proteolytic degradation [Bab, I. *et al* (1992) *EMBO J.* 11:1867].

- C-terminal modified analogues of the osteogenic growth polypeptide, such as [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub>, bind to the OGPBP. The modified analogues do not share the OGP stimulation of cell proliferation and do not react with certain anti-OGP antibodies
- 30 [Israel Patent Application No. 101747]. These polypeptide analogues can be used to release OGP from its complex with an OGPBP. If the competitive reaction takes place in

a tissue culture medium, previously incubated with cells or a biological fluid having peptidase activity, the released OGP becomes exposed to proteolytic degradation resulting from said peptidase activity. Nevertheless, the possibility existed that short peptides resulting from the proteolytic degradation may retain the OGP activity.

- 5            Since the OGP molecule is too large for effective oral administration, it would be of therapeutic importance to find oligopeptides of six or less amino acids residues, that retain the OGP biological activity. such short oligopeptides could be modified into a stable pharmaceutical preparation suitable for oral or other systemic treatment of several pathological conditions, particularly conditions involving loss of bone tissue. In addition,
- 10           the identification of such oligopeptides would be an essential step towards the definition of the minimal amino acid sequence that still retains the OGP activity, which may provide the basis for further drug design.

The present invention indeed relates to such native or synthetic osteogenically active oligopeptides.

- 15           The following abbreviations are used herein:

OGP(s) - osteogenic growth polypeptide(s).

OGPBP(s) - osteogenic growth polypeptide binding protein(s).

irOGP - immunoreactive OGP.

sOGP - synthetic OGP.

## 20    Summary of the Invention

The invention relates to biochemically pure oligopeptides having stimulatory activity on osteoblastic and/or fibroblastic cells having a molecular weight between 200 and 2,000.

- 25           Preferred oligopeptides according to the invention comprise the amino acid sequence: Tyr-Gly-Phe-His-Gly or the amino acid sequence Gly-Phe-Gly-Gly.

Particularly preferred are oligopeptides wherein the five C-terminal amino acid residues are the five or four amino acid residues of said amino acid sequences.

- 30           The invention also relates to a method of isolating a biochemically pure oligopeptide having stimulatory activity on osteoblastic or fibroblastic cells from a biological sample comprising the steps of: (a) discarding from said biological sample peptides having a molecular weight lower than 3000; (b) incubating the medium obtained in step (a) with a polypeptide that binds to an osteogenic growth polypeptide binding protein/s and does not bind to an antibody directed against osteogenic growth polypeptide

- in the presence of protease inhibitors to compete said osteogenic growth polypeptide out from its complex with said osteogenic growth polypeptide binding protein/s; and (c) separating the immunoreactive osteogenic growth peptide from the reaction medium obtained in step (b) by chromatographic methods and to biochemically pure peptides
- 5 having stimulatory activity on osteoblastic or fibroblastic cells prepared by this method.

In addition, the invention relates to pharmaceutical compositions for stimulating the formation of osteoblastic or fibroblastic cells, and consequent bone formation, comprising a therapeutically effective amount of an oligopeptide according to the invention and a pharmaceutically acceptable carrier.

#### 10 Brief Description of the Figures

- Figure 1 shows production of steady-state and total irOGP by osteoblastic ROS 17/2.8 and MC3T3 E1 osteoblastic cells and NIH 3T3 fibroblasts. During the measurement period the cells were grown in chemically defined medium containing 4% BSA. Medium aliquotes for irOGP determination were obtained immediately after the
- 15 addition of a BSA-containing medium ("0" time-point) and at the indicated times thereafter. Steady state and total irOGP were determined as before [Bab *et al* (1992) EMBO J. 11: 1867; Israel Patent Application No. 101747].

- Figure 2 is flow chart describing the purification of irOGP from 24h tissue culture medium prepared as described in Fig. 1 above using MC3T3 E1 cells. Peptides of
- 20 a molecular weight lower than 3000 were separated by three repeated dilution/centrifugation cycles. The retentate was then incubated with [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub> and recentrifuged. The filtrate was collected, and the irOGP separated from the [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub> using reverse phase HPLC and further purified as shown in Fig. 3. The purified irOGP in the peaks seen in Fig. 3 were subjected to amino acid
- 25 sequencing by automated Edman's degradation and tested for proliferative activity as shown in Fig. 4.

- Figure 3 shows HPLC separation of A - irOGP and [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub> using reverse phase Vydac C4 column; and B - the two irOGP peaks, using Merck C18 reverse-phase column. Dashed and continuous lines represent light absorbance and
- 30 immunoreactivity, respectively.

Figure 4 shows the effect of OGP(10-14) (A) and OGP(10-14)His<sup>13</sup> (B) (recovered from the respective B-1 and B-2 peaks of Fig. 3) on osteoblastic MC3T3 E1 cell number *in vitro*. Dashed line - effect of positive SOGP control. Data are mean  $\pm$

SEM of triplicate cultures.

Figure 5 shows the effect of synthetic OGP(10-14) (o---o---o---o---o) and OGP (10-14)His<sup>13</sup> (▲-▲-▲) on osteoblastic MC3T3 E1 (A) and fibroblastic NIH 3T3 (B) cell number *in vitro*. Cell cultures were set and challenged as in Figs. 1 and 4. Cf. effect of positive sOGP control (□-□-□). Data are mean  $\pm$  SEM of triplicate cultures presented as the ratio of treatment to BSA only control T/C ratio).

Figure 6 shows the effect of synthetic Ac-Met<sup>0</sup>OGP(10-14) on osteoblastic MC3T3 E1 (A) and fibroblastic NIH 3T3 (B) cell number *in vitro*. Cell cultures were set and challenged as in Figs. 1 and 4. Dashed line - effect of positive SOGP control. Data are mean  $\pm$  SEM of triplicate cultures presented as the ratio of treatment to BSA only control (T/C ratio).

#### Detailed Description of the Invention

OGP is a 14-residue polypeptide identified from regenerating bone marrow which has been shown to stimulate the proliferation and alkaline phosphatase activity of osteoblastic and fibroblastic cells *in vitro* and to increase bone formation and trabecular bone mass in rats when injected *in vivo*. The amino acid sequence of OGP is as follows:

Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly

Synthetic OGP, with an identical amino acid sequence and biological activity has been prepared by standard solid phase methodology. It has also been found that in different biological fluids OGP forms a complex with OGPBP(s) and that sOGP analogues modified at their C-terminal region can be used to competitively release the total irOGP from OGP-OGPBP complexes, as described above.

The inventors have found that if the competitive reaction takes place in a tissue culture medium, previously incubated with cells or a biological fluid having peptidase activity, the OGP released from the OGP-OGPBP complex becomes exposed to proteolytic degradation by said peptidase activity. Surprisingly, the inventors have found that short oligopeptides, which were found to be present in said competitive reaction medium, retain the stimulatory activity on osteoblastic and fibroblastic cells, and consequently on bone formation.

The invention thus relates to a biochemically pure oligopeptide having stimulatory activity on osteoblastic and/or fibroblastic cells having a molecular weight of 200 to 2,000, preferably 200 to 100:

Oligopeptides which can be specifically mentioned include those having 6 or more

amino acids, preferably 6 to 10 amino acids. Other oligopeptides which can be specifically mentioned are those having 3 or 4 amino acids and those oligopeptides having 5 amino acids other than the pentamer Tyr-Gly-Phe-Gly-Gly.

Preferred oligopeptides according to the invention comprise the amino acid sequence: Tyr-Gly-Phe-His-Gly. Particularly preferred are oligopeptides wherein the five C-terminal amino acid residues are the five amino acid residues of said amino acid sequence. Most preferred is pentapeptide having the formula: Tyr-Gly-Phe-His-Gly. An additional preferred oligopeptide is the tetrapeptide Gly-Phe-Gly-Gly.

The invention also relates to a method of isolating a biochemically pure oligopeptide having stimulatory activity on osteoblastic or fibroblastic cells from a biological sample comprising the steps of: (a) discarding from said biological sample peptides having a molecular weight lower than 3000; (b) incubating the medium obtained in step (a) with a polypeptide that binds to an osteogenic growth polypeptide binding protein/s and does not bind to an antibody directed against osteogenic growth polypeptide in the presence of protease inhibitors to compete said osteogenic growth polypeptide out from its complex with said osteogenic growth polypeptide binding protein/s; and (c) separating the immunoreactive osteogenic growth peptide from the reaction medium obtained in step (b) by chromatographic methods.

The peptides having molecular weights lower than 3000 can be discarded by, for example ultra-filtration with a cut-off of 3000 MW. The protease inhibitors can be commercially available inhibitors, for example E-64, Leupeptide or PMSF or mixtures thereof. The separation of the immunoreactive osteogenic growth peptide in step (c) can be accomplished by available HPLC techniques. A specific embodiment of the method of the invention is described in the Examples.

Biochemically pure peptides having stimulatory activity on osteoblastic or fibroblastic cells prepared by the method according to the invention are also within scope of the present invention. Preferred such peptides are biochemically pure peptides comprising the amino acid sequence: Tyr-Gly-Phe-Gly-Gly or the amino acid sequence: Tyr-Gly-Phe-His-Gly.

Also within the scope of the invention is a hexapeptide having the formula Ac-Met-Tyr-Gly-Phe-Gly-Gly and a tetrapeptide having the formula Gly-Phe-Gly-Gly.

As will be shown in the following Examples, the oligopeptides according to the invention, whether obtained by said method of isolation or synthesized, possess

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stimulatory activity on osteoblastic and fibroblastic cells, and may thus be of great  
therapeutical value. The invention therefore also relates to pharmaceutical compositions  
for stimulating the formation of osteoblastic or fibroblastic cells comprising a  
therapeutically effective amount of an oligopeptide having stimulatory activity on  
5 osteoblastic and/or fibroblastic cells having a molecular weight between 200 and 2000 and  
a pharmaceutically acceptable carrier.

Preferred pharmaceutical compositions according to the invention comprise a  
therapeutically effective amount of an oligopeptide comprising the amino acid sequence  
Tyr-Gly-Phe-His-Gly, Tyr-Gly-Phe-Gly-Gly or Gly-Phe-Gly-Gly or a mixture thereof and  
10 a pharmaceutically acceptable carrier.

The pharmaceutical compositions of the invention may be particularly useful for  
the stimulation of osteoblastic cells and/or fibroblastic cells, and consequently enhanced  
bone formation in various pathological conditions, for example osteoporosis (or osteopenia  
of any etiology), fracture repair, healing of wounds, intraosseous implants and bone  
15 supplementation, or other conditions requiring enhanced bone formation.

The magnitude of a therapeutic dose of a polypeptide of the invention will of  
course vary with the group of patients (age, sex, etc.), the nature of the condition to be  
treated and with the particular polypeptide employed and its route of administration. In  
any case the therapeutic dose will be determined by the attending physician.

20 Any suitable route of administration may be employed for providing a mammal,  
especially a human, with an effective dosage of a polypeptide of this invention.  
Intravenous and oral administration may be preferred.

The pharmaceutical compositions of the invention can be prepared in dosage units  
forms. The dosage forms may also include sustained release devices. The compositions  
25 may be prepared by any of the methods well-known in the art of pharmacy.

The pharmaceutical compositions of the invention comprise as active ingredient  
an oligopeptide of this invention or a mixture of such oligopeptides in a pharmaceutically  
acceptable carrier, excipient or stabilizer, and optionally other therapeutic constituents.  
Acceptable carriers, excipients or stabilizers are non-toxic to recipients at the dosages and  
30 concentrations employed, and include buffers, such as phosphate buffered saline and like  
physiologically acceptable buffers, and more generally all suitable carriers, excipients and  
stabilizers known in the art.



**EXAMPLES****Example 1 - Purification and characterization of pentapeptides from tissue culture medium****Materials**

- 5 Tissue culture ingredients were purchased from Biological Industries, Beit Haemek, Israel. Culture dishes were from Nunc, Roskilde, Denmark. bovine serum albumin (BSA), protease inhibitors and N-ethylmaleimide (NEM) were from Sigma Chemical Co., St. Louis, MO; Cat No. A-7030. Centricon-3 microconcentrators were purchased from Amicon, Inc. Beverly, MA. t-Boc-Gly OCH<sub>2</sub>-Pam resin, N-Box protected amino acid
- 10 derivatives, N,N-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Applied Biosystems Inc. (Foster City, Ca). Hydrogen fluoride (HF) was purchased from Matheson (Secaucus, NJ), Boc-3-I-Tyr(BZ1)-OH from Bachem (Torrance Ca), p-Cresol from Aldrich Chemical Co.
- 15 (Milwaukee, WI) and Sephadex G15F from Pharmacia (Uppsala, Sweden). C18 reverse-phase column and acetonitrile were from E. Merck, Darmstadt, Germany. C4 reverse-phase column was from The Separation Group, Hesperia CA.

**Methods****20 Measuring irOGP accumulation in tissue culture media**

- ROS 17/2.8 or MC3T3 E1 osteoblastic cells or NIH 3T3 fibroblasts were maintained in  $\alpha$ -Minimal Essential Medium supplemented with 10% fetal calf serum (FCS) and subcultured twice a week. Cells for experiments were derived from maintenance cultures at confluency. For the experiment the cells were seeded in 25 cm<sup>2</sup> tissue culture flasks at
- 25 1 x 10<sup>4</sup> cells/cm<sup>2</sup>. The cultures were incubated at 37°C in CO<sub>2</sub> air. For the initial 46 h the medium was supplemented with 10% FCS and 0.2% nucleosides/ribonucleosides followed by an additional 2 h starvation period under serum free conditions. Then the serum free medium was replaced by 8 ml medium containing 4% BSA. Half millilitre medium aliquots for irOGP determination were obtained immediately after the addition of
- 30 BSA containing medium ("0" time-point) and 12, 24, 36 and 48 hrs thereafter. Steady state and total irOGP were determined in these aliquots as before [Bab *et al.* (1992) EMBO J. 11:1867; Israel Patent Application No. 101747].

**Separation between irOGP and OGPBP**

Fig. 2 is a schematic demonstration of the separation between the irOGP and the OGPBP. A 3.75 ml sample of medium collected 24 h after cell starvation was diluted (1:1) with an equal amount phosphate buffered saline (PBS). The diluted medium was centrifuged in multiple centricon-3 microconcentrators (Amicon, Inc., Beverly, MA) for 1.5 h at 5,000 x g. Polypeptides smaller than 3000 MW were washed out of the retentate by three repeated dilution/centrifugation cycles using 1 mM sodium azide in 165 mM ammonium acetate pH 7.0 as diluent. The minimal retentate volume allowed in each micro concentrator was 250  $\mu$ l. To release irOGP from the OGP-OGPBP complex, the 1:1 diluted retentate was incubated for 30 min at 37°C with 450 nmol/ml [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub>, prepared as before [Israel Patent Application No. 101747] in 165 mM ammonium acetate containing 50  $\mu$ M E-64, 50  $\mu$ M Leupeptin and 500  $\mu$ M PMSF and recentrifuged.

**Separation between irOGP and [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub>**

The filtrate obtained by the microconcentration step was partially evaporated to final volume of 600  $\mu$ l. The irOGP content of the filtrate was 0.31 nmol. The filtrate was divided into three equal aliquots. The irOGP in each of these aliquots was separated from the [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub> by HPLC using a reverse phase Vydac protein C4 column employing the following acetonitrile gradient run at a flow rate of 1 ml/min: 3 ml 12% acetonitrile; and 30 ml 12-19% acetonitrile.

**Separation between two irOGP peaks**

Half ml fractions comprising the main irOGP peak recovered from the C4 column were pooled and partially evaporated to final volume of 400  $\mu$ l. The irOGP content of this peak was 0.26 nmol. The filtrate was divided into two equal aliquots. The irOGP in each of these aliquots was subjected to HPLC on a reverse-phase C18 column employing the following acetonitrile gradient run at a flow rate of 1/ml/min: 3 ml 14% acetonitrile and 30 ml 14-19% acetonitrile.

30

**Amino acid sequence determination**

Protein in the two irOGP peaks recovered from the C18 column was subjected to automated peptide sequence analysis in an Applied Biosystems 470A sequencer using the

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program and reagents supplied by the manufacturer. Released amino acid derivatives were identified with the aid of an on-line HPLC system.

### Results

- 5 Fig. 1 shows that in all three cell systems studies, the total irOGP reached a peak 24 h after starvation and exposure to the chemically defined medium. This time period coincides with the confluency of the cultures. During the next 24 h there was an approximately 50% decrease in the total irOGP. The steady state irOGP showed a more or less linear increase during the whole detection period. These results indicate that
- 10 stromal cells produce irOGP and the OGPBP. The rate of synthesis of the irOGP and/or the OGPBP decreases when the cultures reach confluency.

The 24 h culture medium from the MCT3 E1 cells was subjected to repeated dilution/centrifugation cycles to remove the non-bound irOGP and other peptides with

15 molecular weight smaller than 3000 (Fig. 2). Only 0.96% of the total irOGP could be discarded in this way. The remaining irOGP was left in the form of OGP-OGPBP complex that was contained in the retentate because of its high molecular weight. (Table A). The HPLC separation of [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub> resulted in three major light absorption peaks. The greater of these peaks eluted at 19.5-23.5 min retention time, a

20 position similar to that of [Cys<sup>15</sup>(NEM)]OGP-NH<sub>2</sub> run separately under the same conditions. This peak did not show OGP immunoreactivity. Both other peaks were only partially separated from each other and showed a high irOGP content (Fig. 1A), 11.5% that of the total irOGP present in the culture medium (Table A). It is likely that most of this decrease in irOGP content resulted from peptidase activity during the displacement

25 reaction, in spite of the presence of peptidase inhibitors. To enhance the separation between these irOGP peaks the respective fractions (retention time 11-12.5 min) were pooled and subjected to a second HPLC step that resulted in two distinct light absorption peaks, both showing high irOGP content (Fig. 1B). These peaks were designated B-1 and B-2.

30

**TABLE A**  
**RECOVERY OF IMMUNOREACTIVE OGP FROM**  
**INDIVIDUAL PURIFICATION STEPS**

5	<div style="display: flex; justify-content: space-between;"> <span>irOGP</span> <span>percent</span> </div> Purification step (total nmol in preparation) recovery	
10	24-h post starvation medium 270.0	100
	Initial ultrafiltration	0.96
	Post-displacement ultrafiltration 31.0	11.5
	Reverse-phase HPLC	4.7

- 15 The respective fractions eluted with retention times 23.5-24 min and 25-25.5 min were pooled. Aliquots of the pooled fractions were allocated for testing their proliferative effect in the MC3T3 E1 cell assay. Amino acid sequencing revealed that peak B-1 contained a pentapeptide identical to the C-terminal region (residues 10-14) of OGP. Peak B-2 contained a similar pentapeptide in which Gly<sup>13</sup> of the OGP was substituted by His
- 20 (Table B)

**TABLE B**  
**AMINO ACID SEQUENCES OF PENTAPEPTIDES**

- |    |           |                     |
|----|-----------|---------------------|
| 25 | Peak B-1* | Tyr-Gly-Phe-Gly-Gly |
|    | Peak B-2* | Tyr-Gly-Phe-His-Gly |

\*Defined in Figure 3.

The proliferative activity of either peak in the MC3T3 E1 cell assay was identical to that of positive sOGP controls with a peak at 10<sup>-13</sup>M (Fig 4).

30

#### Example 2- Activity of Synthetic Pentapeptides

##### Peptide synthesis

Synthetic peptides of this invention were prepared by solid phase method of Merrifield [Merrifield, (1969) Adv. Enzymol. 32: 221] using an Applied biosystems Model 430A

Automated peptide Synthesizer (Applied Biosystems Inc., Foster City, CA). In the case of peptide free acid the synthesis was carried out on 0.5 mmol t-Boc-Gly-PAM resin (1% cross-linked, 0.61 meq/g). t-Boc- Gly-MBHA resin (1% cross-linked, 0.66 meq/g) was used for the amidated tetrapeptide. The amino acid derivatives were protected on the  $\alpha$ -amino function by t-butyloxycarbonyl (Boc) groups. Protection of the tyrosine side chain was by Z. The amino acid derivatives were coupled via the DCC-mediated preformed symmetrical anhydride method of Hagemaiier, H. and Frank, H. [Hoppe-Seyler's Z. (1972) *Physiol. Chem.* 353:1973]. The coupling of each amino acid residue was repeated twice. Deprotection of the blocked amino terminus was by treatment with 25% TFA in DCM. Side chains were deprotected and the peptide cleaved from the resin using the HF procedure where a mixture of 4 ml anisole and 36 ml liquid HF was used for 75 min at 0°C. The crude synthetic peptides were purified on a Merck-Hitachi 655A-11 HPLC instrument equipped with a Waters  $\mu$  BondPark™ C18 column (1.9x15.0 cm). The cartridge was pumped with acetonitrile linear gradients containing TFA (Table C) at a flow rate of 6.0 ml/min.

The BH-OGP (11-14) (BH = Bolton-Hunter) was prepared by reacting 3-(3-iodo-4-hydroxyphenyl) propionic acid N-hydroxysuccinimidyl ester with purified OGP (11-14) using the method of Michelot *et al* [Michelot R. *et al* (1980) *Biochem. Biophys. Res. Comm.* 95:491-498].

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TABLE C

**ACETONITRILE LINEAR GRADIENTS USED FOR HPLC PURIFICATION  
OF DI-, TRI-, TETRA-, PENTA- AND HEXA-OGP RELATED PEPTIDES**

		<u>Gradient</u>		
<b>5</b>	<b><u>Peptide</u></b>	<b><u>(% Acetonitrile)</u></b>	<b><u>Time (min)</u></b>	<b><u>% TFA</u></b>
	OGP (13-14)	0% isocratic	30	.1
	OGP (11-12)	0% isocratic	30	.1
	OGP (12-14)	0% isocratic	30	.1
	OGP (11-13)	0% isocratic	30	.1
<b>10</b>	OGP (10-12)	0-25	120	.1
	OGP (11-14)	0-30	120	.1
	BH*-OGP(11-14)	0-25	90	.1
	OGP(11-14)NH <sub>2</sub>	0-5	90	.05
	OGP(10-13)	0-25	120	.1
<b>15</b>	OGP(10-14)	0-20	100	.1
	OGP(10-14)His <sup>13</sup>	0-20	100	.1
	Ac-Met <sup>0</sup> [OGP(10-14)]	0-20	150	.1

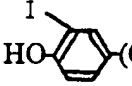
\*BH = Bolton-Hunter reagent

20

Table D demonstrates the conversion between the nomenclature used herein and amino acid sequence of the peptides.

TABLE D

**5 AMINO ACID COMPOSITION OF SYNTHETIC  
DI-, TRI-, TETRA-, PENTA- AND HEXA- OGP RELATED PEPTIDES**

	OGP (13-14)	Gly-Gly
	OGP (11-12)	Gly-Phe
<b>10</b>	OGP (12-14)	Phe-Gly-Gly
	OGP (11-13)	Gly-Phe-Gly
	OGP (10-12)	Tyr-Gly-Phe
	OGP (11-14)	Gly-Phe-Gly-Gly
<b>15</b>	BH*-OGP(11-14)	 (CH <sub>2</sub> ) <sub>2</sub> CO-Gly-Phe-Gly-Gly
	OGP (11-14) NH <sub>2</sub>	Gly-Phe-Gly-Gly-NH <sub>2</sub>
	OGP (10-13)	Tyr-Gly-Phe-Gly
	OGP (10-14)	Tyr-Gly-Phe-Gly-Gly
	OGP (10-14) His	Tyr-Gly-Phe-His-Gly
<b>20</b>	Ac-Met <sup>0</sup> [OGP(10-14)]	Ac-Met-Tyr-Gly-Phe-Gly-Gly

\*BH = Bolton-Hunter reagent

### Results

The proliferative effect of either pentapeptide in the MC 3T3 E1 and NIH 3T3 cell assays  
**25** was similar to that of sOGP positive controls. The respective peak activities were at 10<sup>-13</sup>  
 and 10<sup>-11</sup>-10<sup>-10</sup>M peptide concentration (Fig. 5).

### Example 3 - Proliferative effect of hexapeptide

The hexapeptide of Ac-Met<sup>0</sup>[OGP](10-14)] was prepared for testing the feasibility of using  
**30** Ac-[<sup>35</sup>S]Met[OGP(10-14)] for metabolic and binding studies.

### Results

The Ac-Met<sup>0</sup>[OGP](10-14)] stimulated the MC 3T3 E1 and NIH 3T3 cell number in a

manner closely similar to that of positive sOGP controls with peaks at  $10^{-13}$  and  $10^{-11}$  M peptide concentration, respectively (Fig 6).

#### Example 4 - Proliferative effect of tetra-, tri- and di-peptides

- 5 All the five tetrapeptides had a dose-response effect on the MC 3T3 E1 and NIH 3T3 cell number with peaks at  $10^{-13}$  and  $10^{-11}$  M peptide concentration, respectively. The magnitude of the BH-OGP (11-14) peak effect in either cell system was highest and similar to that of the SOGP positive control. The peak activity of the OGP(11-14), OGP(10-13) and OGP (10-12) showed intermediate values. The OGP (11-14)  $\text{NH}_2$  10 showed the lowest peak effect. OGP (12-14), OGP (11-13), OGP(13-14) and OGP (11-12) did not affect the MC 3T3 E1 and NIH 3T3 cell number (Table E).

TABLE E  
PROLIFERATIVE ACTIVITY OF SYNTHETIC  
DI-, TRI- AND TETRA-OGP RELATED PEPTIDES  
{Percent Activity of Full Length Peptide [OGP(1-14)]}

		<u>MC3T3 E1</u>			<u>NIH 3T3</u>		
				No. of			No. of.
20	<u>Peptide</u>	<u>Mean</u>	<u>Range</u>	<u>Expers.</u>	<u>Mean</u>	<u>Range</u>	<u>Expers</u>
	BH*-OGP(11-14)	113	70-137	3	80	63-112	3
	OGP(11-14)	66	34-100	8	68	34-100	4
	OGP(10-13)	58	42-74	2	89	89	1
	OGP(10-12)	41	41	1	57	57	1
25	OGP(11-14) $\text{NH}_2$	25	19-31	2	33	31-35	2
	OGP(12-14)	0	0	3	0	0	3
	OGP(11-13)	0	0	3	0	0	3
	OGP(13-14)	0	0	3	0	0	3
	<u>OGP(11-12)</u>	0	0	3	0	0	3

30

\*BH - Bolton-Hunter reagent.



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CLAIMS

1. A biochemically pure oligopeptide having stimulatory activity on osteoblastic and/or fibroblastic cells having a molecular weight of 200 to 2,000.
2. An oligopeptide according to claim 1 having a molecular weight of 200 to 1,000.
3. An oligopeptide according to claim 1 comprising the amino acid sequence:  
Tyr-Gly-Phe-His-Gly.
4. An oligopeptide according to claim 3 wherein the five C-terminal amino acid residues are the five amino acid residues of said amino acid sequence.
5. A pentapeptide according to claim 4 having the formula:  
Tyr-Gly-Phe-His-Gly.
6. An oligopeptide according to claim 1 comprising the amino acid sequence  
Gly-Phe-Gly-Gly.
7. An oligopeptide according to claim 6 wherein the four C-terminal amino acid residues are the four amino acid residues of said amino acid sequence.
8. A tetrapeptide according to claim 7 having the formula:  
Gly-Phe-Gly-Gly.
9. A hexapeptide according to claim 1 having the formula:  
Ac-Met-Tyr-Gly-Phe-Gly-Gly.
10. A method of isolating a biochemically pure oligopeptide having stimulatory activity on osteoblastic or fibroblastic cells from a biological sample comprising the

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steps of:

(a) discarding from said biological sample peptides having a molecular weight lower than 3000; to leave a medium.

(b) incubating the medium obtained in step (a) with a polypeptide that binds to an osteogenic growth polypeptide binding protein/s and does not bind to an antibody directed against osteogenic growth polypeptide in the presence of protease inhibitors to compete said osteogenic growth polypeptide out from its complex with said osteogenic growth polypeptide binding protein/s; and

(c) separating the immunoreactive osteogenic growth peptide from the reaction medium obtained in step (b) by chromatographic methods.

11. A method according to claim 10 wherein in step (a) the peptides having molecular weights lower than 3000 are discarded by ultra-filtration with a cut-off of 3000MW.

12. A method according to claim 10 or claim 11 wherein said protease inhibitors are E-64, Leupeptin or PMSF or mixtures thereof.

13. A method according to any one of claims 10 to 12 wherein in step (c) the immunoreactive osteogenic growth peptide is separated by HPLC.

14. Biochemically pure peptides having stimulatory activity on osteoblastic or fibroblastic cells prepared by the method according to any one of claims 10 to 13.

15. Biochemically pure peptides according to claim 14 comprising the amino acid sequence: Tyr-Gly-Phe-Gly-Gly.

16. Biochemically pure peptides according to claim 14 comprising the amino acid sequence: Tyr-Gly-Phe-His-Gly.

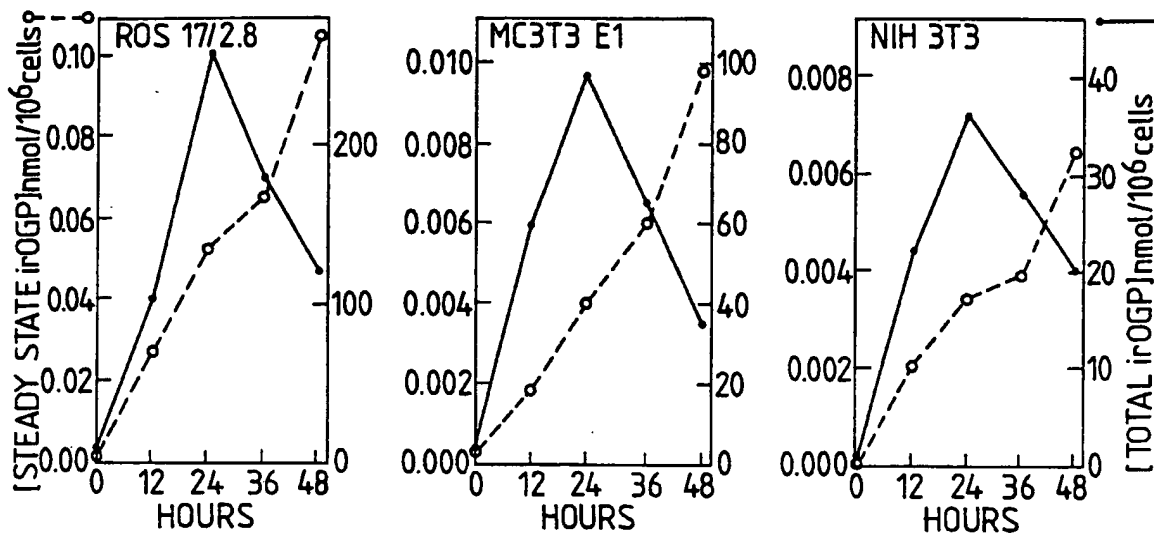
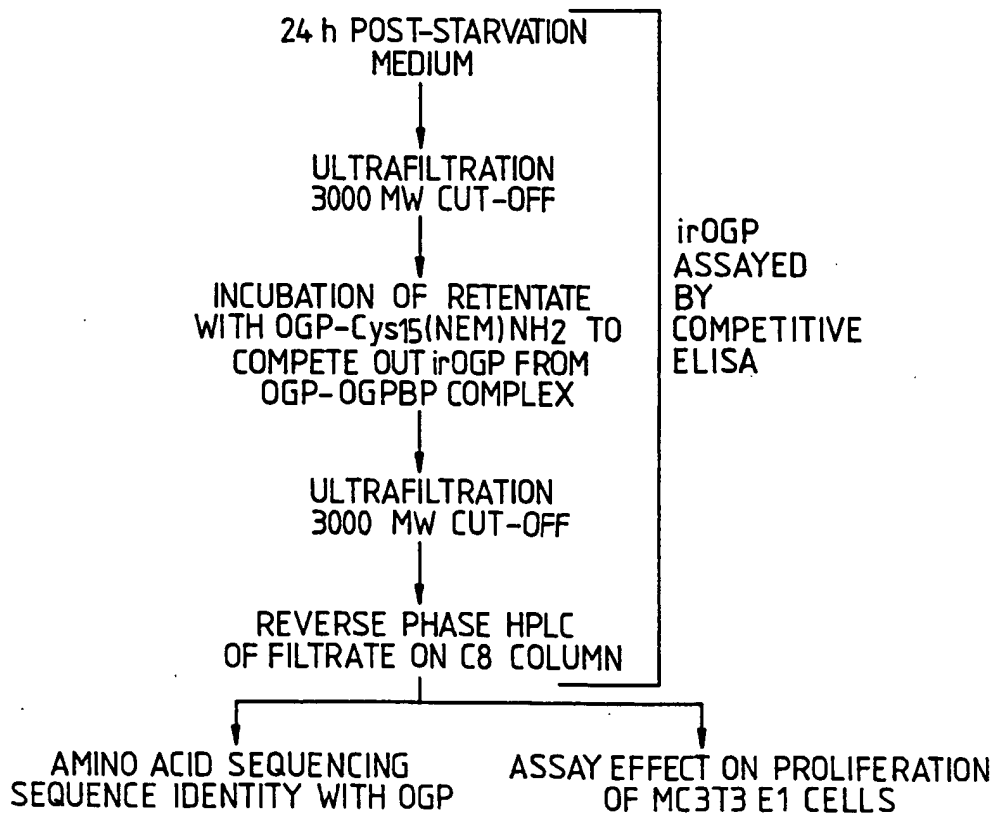
- 17 -

17. Biochemically pure peptides according to claim 14 comprising the amino acid sequence of Gly-Phe-Gly-Gly.
18. A pharmaceutical composition comprising an oligopeptide according to any one of claims 1 to 9 or 14 to 17 and a pharmaceutically acceptable carrier.
19. A method of preparing a pharmaceutical composition comprising formulating an oligopeptide according to any one of claims 1 to 9 or 14 to 17 with a pharmaceutically acceptable carrier.
20. An oligopeptide according to any one of claims 1 to 9 or 14 to 17 for use in a method of treatment of the human or animal body by surgery or therapy or in diagnosis.
21. An oligopeptide according to claim 20 wherein the method of treatment is for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, and other conditions requiring enhanced bone formation cells.
22. The use of an oligopeptide according to any one of claims 1 to 9 or 14 to 17 in the manufacture of a medicament for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, and other conditions requiring enhanced bone formation cells.
23. A method of treatment of a human or animal patient for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, and other conditions requiring enhanced bone formation

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cells which comprises administering to the human or animal patient a therapeutically effective amount of an oligopeptide according to any one of claims 1 to 9 or 14 to 17.

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*Fig.1.**Fig.2.*

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Fig. 3.

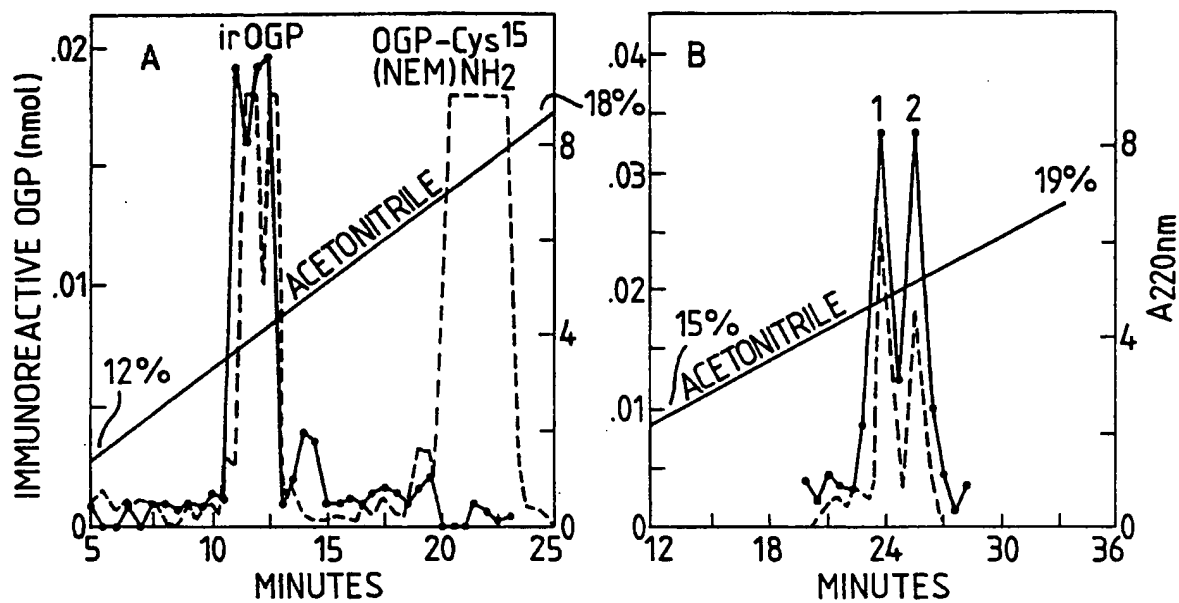
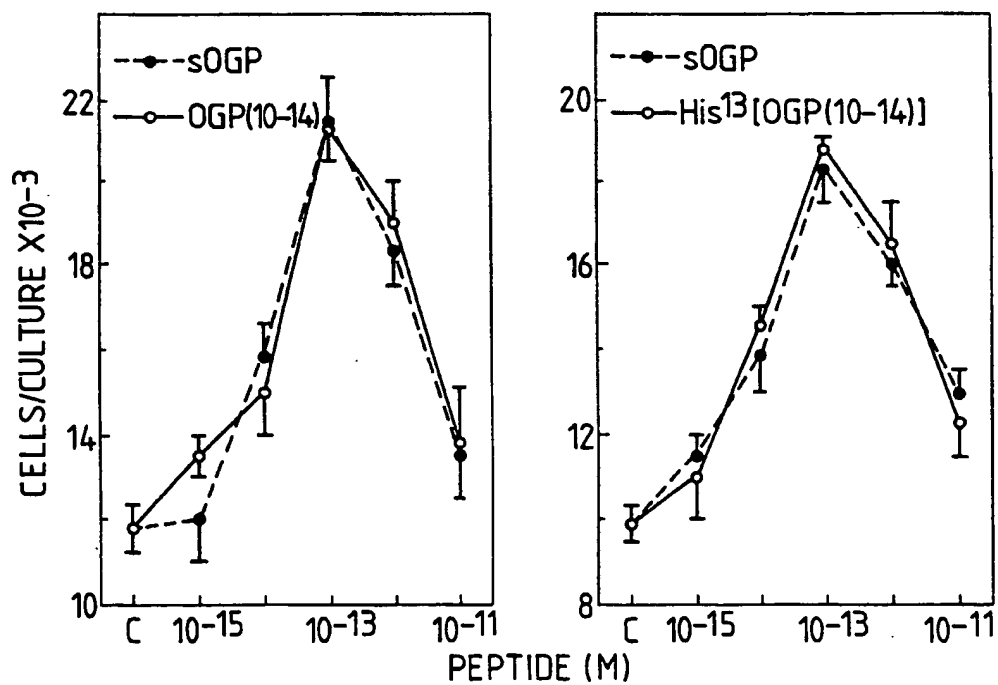


Fig. 4.



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Fig. 5.

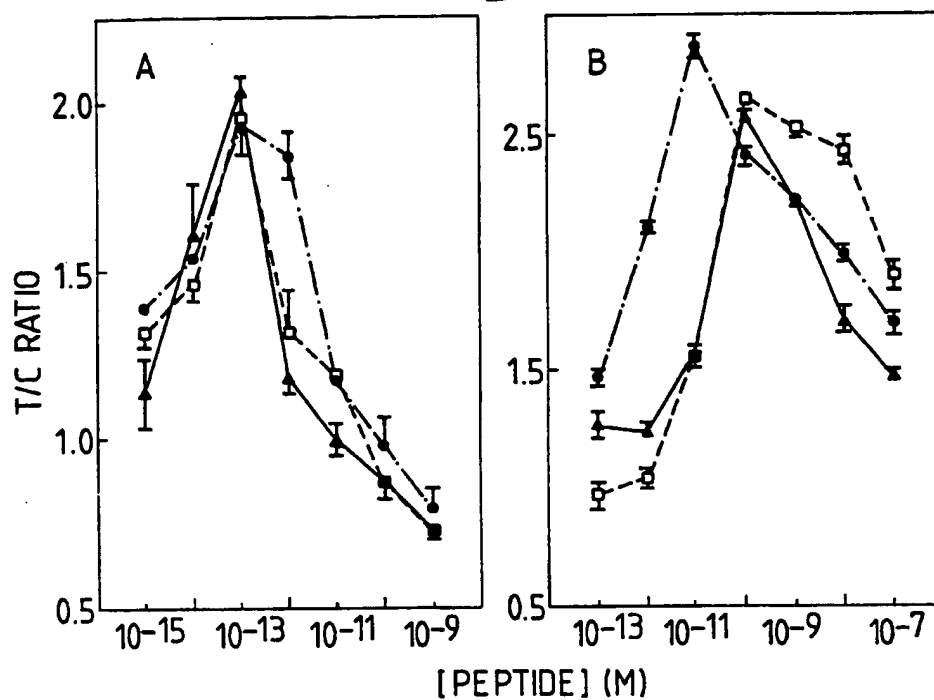
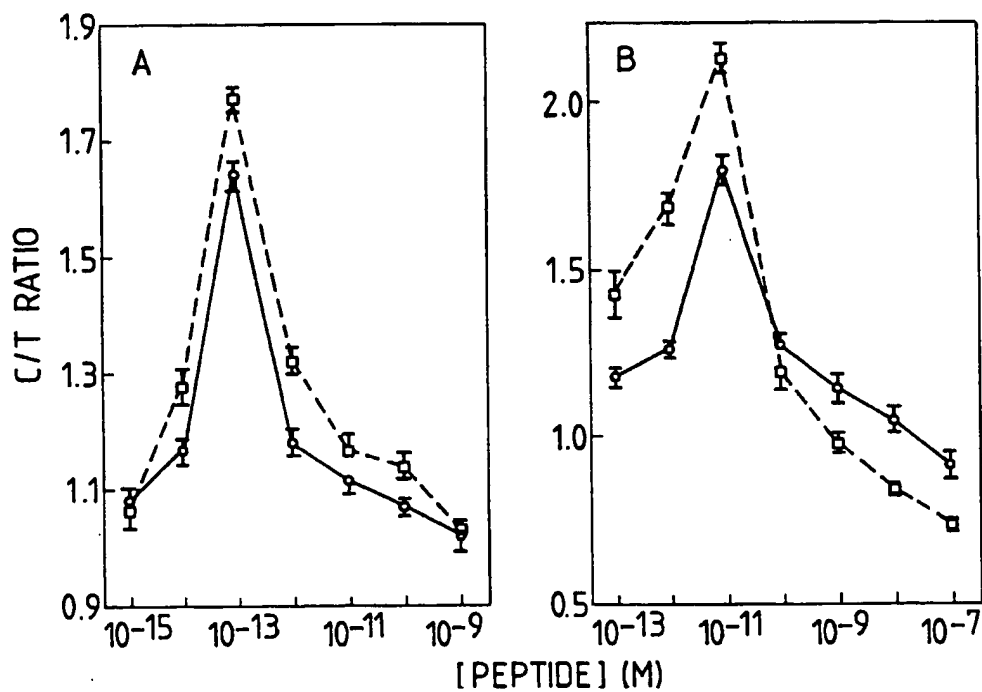


Fig. 6.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/00416

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K5/10 C07K7/06 C07K7/08 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO JOURNAL vol. 11, no. 5 , May 1992 , EYNSHAM, OXFORD GB pages 1867 - 1873 I. BAB ET AL. 'Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity' see page 1869, left column, paragraph 2 - page 1870, left column, paragraph 1; table I see page 1871, right column, paragraph 2 see page 1872, left column, paragraph 4 -paragraph 5  --- -/--	1,2,6,7, 14,15, 17-23

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&amp;\* document member of the same patent family

Date of the actual completion of the international search

15 July 1994

Date of mailing of the international search report

22.07.94

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 94/00416

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 110, no. 21, 22 May 1989, Columbus, Ohio, US; abstract no. 186143, E.P. KHARCHENKO ET AL. 'Highly active analogs of apiate-like peptides historphin and kapporphine' page 96 ;column L ; see abstract & VOPR. MED. KHIM. vol. 35, no. 2 , 1989 pages 106 - 109 cited in the application ----	1,2,6,7, 14,15, 17-23
X	EP,A,0 384 731 (MERCK & CO. INC.) 29 August 1990 cited in the application see page 3, line 18 - line 26; claims; example 4; table A ----	1,2,6,7, 14,15, 17-23
P,X	EP,A,0 572 122 (YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY) 1 December 1993 see claims; examples ----	1,6,7, 10,14, 15,17-23
P,X	BIOCHIMICA ET BIOPHYSICA ACTA vol. 1178, no. 3 , 13 October 1993 pages 273 - 280 Z. GREENBERG ET AL. 'Mitogenic action of osteogenic growth peptide (OGP): role of amino and carboxy-terminal regions in charge' see Discussion on pages 279-280 ----	1
A	'Preparation and Bioassay of Bone Morphogenic Protein and Polypeptide Fragments', by M.R. Urist et al., in Methods in Enzymology Vol. 146 ed. by. D. Barnes and D.A. Sirbasku, pub. Academic Press, 1987 pages 294-312 see page 302, paragraph 3 -----	1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB94/00416

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claim 23 is directed to a method of treatment of  
(diagnostic method practised on) the human/animal body the search has  
been carried out and based on the alleged effects of the compound/  
composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such  
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 94/00416

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0384731	29-08-90	CA-A, C 2010660 JP-A- 2282396	23-08-90 19-11-90
EP-A-0572122	01-12-93	NONE	